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CORROSION2001

APPLICATION OF ENVIRONMENTAL CELL TRANSMISSION ELECTRON MICROSCOPY TO MICROBIOLOGICALLY INFLUENCED CORROSION

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ABSTRACT

The spatial/chemical relationship between bacteria, their biofilms, and metal substrata was examined in an environmental cell transmission electron microscope equipped with an energy loss spectrometer. The advantage of environmental cell transmission electron microscopy is that unfixed, hydrated specimens can be examined, in more or less their natural state, with high spatial resolution.

INTRODUCTION

Microbiologically influenced corrosion (MIC) has been studied by transmission electron microscopy (TEM). Localization of bacteria on metal surfaces and their distribution throughout corrosion products have been examined.[1-3] Copper immobilization by bacterial biofilms during microbial colonization of copper surfaces has been demonstrated.[2] Bacteria were arranged in parallel sheets between layers of corrosion products on a copper-nickel (90:10) alloy.[3] In that work, it is reported that bacteria can be associated with copper in several different ways: copper can be bound in extracellular mucilages, corrosion products and copper particles can be encrusted on cell walls or impregnate cell interiors. However, the study of MIC by conventional TEM is limited because the techniques of fixation, dehydration, embedding, and microtoming, necessary to prepare thin specimens, are known to alter delicate cellular structures and introduce artifacts. Fixation and dehydration preserve some of the structure, but buffer and organic solvent washes remove soluble ions from extracellular and intracellular sources. Furthermore, dehydration causes extracellular polymeric substances (EPS) to collapse into a web-like biofilm network. One technique to preserve EPS morphology is high-pressure freezing (HPF) and freeze substitution.[4] However, HPF is suitable for small samples and not adaptable for larger samples normally of interest in corrosion.

Environmental scanning electron microscopy (ESEM) has provided valuable data on dehydration artifacts[5] and has advanced the study of hydrated microorganisms as well as marine aggregates.[6-8] Environmental cell (EC)-TEM would clearly be advantageous because unfixed, hydrated

bacteria can be examined, in more or less their natural state, in association with corroding metals at high spatial resolution. Here we report the preliminary results of an EC-TEM study of *Pseudomonas* with corroding iron particles and *Oceanospirillum* with corroding copper. We show the capability of performing elemental analysis with electron energy loss spectroscopy (EELS) and the potential for oxidation state determination in an EC- transmission electron microscope.

METHODS

Examination by EC-TEM and Conventional TEM

In this study, a JEOL JEM-3010 transmission electron microscope operating at 300 keV was used. It is equipped with a JEOL EC system which has interchangeable EC specimen holders, an energy dispersive x-ray spectrometer (EDXS), and a Gatan imaging filter (GIF200) capable of EELS measurements. The microscope was equipped with two EC specimen holders: a two-line gas EC and a four-line gas/liquid EC. These specimen holders are connected to the EC system by flexible, stainless steel lines. Both *in situ* holders are capable of circulating dry or water saturated gas, the latter enables the examination of hydrated specimens (gas circulation is supported by two lines). The four-line holder has two additional lines which can be used to independently inject several microliters of two different liquids. Each EC holder consists of a small cylindrical cell sealed by two electron transparent windows on the top and bottom (see Figure 1 for the 4-line cell used in this study). The windows were fabricated from 15 - 20 nm thick amorphous carbon (a-C) films that cover seven hexagonally arrayed, 0.15 mm apertures on a 3.5 mm diameter Cu disk (see Figures 2 and 3). Prior to use, the windowed grids were tested to withstand a pressure differential of 250 torr for one minute. The a-C films, if produced properly, can be strong enough to sustain a pressure differential up to 1/2 atmosphere. A computer controls the EC system (Figure 4) and facilitates the operation of inserting and removing the EC holder from the microscope column without breaching the delicate windows.

In this study, the EC was operated at 100 torr with room air (saturated with water vapor) circulating through the cell at a rate of $\approx$ 2 liter/min. Specimens were supported on the lower a-C film window. The microscope was also equipped with conventional single- and double-tilt specimen holders for conventional TEM. The single-tilt holder has an adapter which accepts windowed EC grids for analysis following their use in the EC holder.

Copper in Natural Seawater

Copper foils (99.9% pure, 150-microns thick) were cut into tapering triangles (1.5 cm tall triangle with a 0.5 - 1.0 cm base). Triangular tips were sandwiched between glass plates and pressed overnight to straighten. Triangles were degreased in acetone and cleaned in 100% ethanol. Foils were attached to a glass base with a silicone adhesive and placed in flowing, aerated Key West seawater for 30 days. After exposure, foils were fixed in 4% gluteraldehyde in 0.2 μm filtered seawater at 4° C overnight and processed through a graded series of filtered seawater/distilled water, and finally into distilled water. Foil samples were dehydrated through a graded water/ethanol series, followed with an acetone wash and embedded into Spurr's resin (Electron Microscopy Sciences, Ft. Washington, PA). Samples were sectioned on a Leica Ultracut UCT ultramicrotome, and the resulting 100 nm sections examined by conventional TEM using EELS.

Pseudomonas putida / Iron System

Pseudomonas putida (P. putida), provided by Dr. J. Jones-Meehan (Naval Research Laboratory, DC) was maintained in brain heart infusion broth at room temperature and the cells were transferred to fresh medium every two days. Fresh log phase cultures were washed three times with distilled water and placed onto an a-C coated slot grid and examined by conventional TEM under high vacuum.

Iron filings were prepared from carbon steel (C1010) by light sanding with 600 grit abrasive paper and isolated from the abrasive grit using a Teflon® coated magnet. Iron filings were then degreased with acetone, washed with ethanol, and placed into sterile distilled water. Filings were allowed to corrode (~ 3 days) until corrosion products were visible with a dissecting microscope. Fresh log phase cultures of *P. putida* (maintained as above) were washed three times with distilled water, placed into a tube containing corroding iron particles, and allowed to incubated over night on a rotator at room temperature. An aliquot of solution containing suspended iron particles and bacteria were placed onto the a-C film of the lower EC windowed grid. Particles were allowed to settle for 5 minutes and excess solution was wicked away with filter paper. The EC chamber was sealed and inserted in the column of the microscope and examined under an atmosphere of 100 torr of saturated water vapor.

Oceanospirillum / Copper System

Oceanospirillum sp. from Dr. D. White (Oak Ridge National Laboratories, Oak Ridge, TN) was maintained in marine broth at room temperature. Copper filings were prepared from 99.9% pure copper foils by sanding with 600 grit sand paper, collected by rinsing into a test tube with distilled water, degreased with acetone, washed with ethanol, and placed into Key West seawater that had been filtered (0.2 μm pore filter size) and pasteurized (70° C for 30 minutes). Filings were allowed to corrode overnight. Fresh log phase cells were washed three times in sterile seawater and placed into a tube containing corroding copper filings and incubated overnight on a rotator at room temperature. For EC-TEM analysis, the filings/bacteria were centrifuged, supernatant was removed, and the precipitate rinsed with dilute seawater then distilled water. This was repeated again using distilled water to remove trace salts. An aliquot of solution containing suspended copper particles and bacteria was loaded into the EC specimen holder (as outlined above) and examined under an atmosphere of 100 torr of saturated water vapor.

RESULTS

Conventional TEM and EELS were used to examine corrosion products on 99% pure copper foils exposed to natural seawater containing indigenous microorganisms (Figure 5). For the determination of the oxidation state of metal atoms at high spatial resolution, techniques using EELS have been previously developed.[9-12] Basically, these techniques compare the differences in fine structure of $L_{2,3}$ absorption edges (dominated by excited electron dipole transitions between the core $2p_{3/2}$ and $2p_{1/2}$ spin-orbit split levels to unoccupied 3d levels) of metals in standards of known oxidation state. Spectra of different $3d^n$ configurations differ and can be used to identify the valency (related to the number of holes in the 3d levels) of an element. For copper metal, the 3d level is filled and consequently the edge at 931 eV has a flat plateau. In comparison, Cu^{+2} has one unoccupied state (hole) in the 3d level allowing transitions and the presence of a peak at the 931 eV edge[13]. As show in Figure 5, the surface of the copper metal exposed to natural seawater is progressively oxidized as evident by the increase in the 931 eV peak in going from the bulk metal to the surface.

Examination of viable *P. putida* deposited on an a-C coated TEM grid placed directly into the microscope column under high vacuum show the typical rod-shaped bacteria, however damage is readily apparent (Figure 6). Internal structures such as the nucleoid and other electron dense structures are visible, however cell membranes are often ruptured by decompression under high vacuum in the column. Furthermore, EPS quickly become filamentous and web-like as they dehydrate under the electron beam.

In comparison, when viable *P. putida* is examined in the EC at 100 torr, under a circulation of air saturated with water vapor, these artifacts are not observed (Figure 7). Cells remain plump/hydrated and the EPS retain moisture and appear as a continuous layer. In Figure 7, examples are shown where *P. putida* is not in direct contact with the corroding particle, rather a thick layer of EPS is found between the bacterium and the corroding particle. Although decompression and dehydration damage is avoided in the EC, electron beam damage of viable *P. putida* is observed within several minutes of electron beam exposure (Figure 8). Degradation results from destruction of weak Van der Waals biomolecular bonds when ionizing energy is transferred by inelastic electron scattering. Earlier studies[14-16] demonstrated that amino acids and bases of nucleic acids show effects of irradiation damage for doses as low as 1.5×10^{-3} to 6×10^{-1} C-cm^{-2}. In Figure 8, progressive damage to a cell nucleoid is readily apparent.

If the water-saturated gas circulating through the EC is replaced with circulating room air, excess moisture in EPS is removed and image resolution is improved (Figure 9). Dehydrating the EPS by circulating (or injecting bursts of) room air improves spectra acquired by EELS. In this study, *P. putida* are often observed encrusted with electron beam dense material which EELS identified as iron rich (Figure 10). For iron, the differences in core-loss fine structure produced by changes in oxidation state are not as conspicuous as with copper. Before the oxidation state of the iron associated with the cells can be determined, a series of iron mineral standards, each with well defined single iron valance state, need to be measured. These measurements are currently being performed.

As shown with *P. putida,* when hydrated *Oceanospirillum* together with corroding copper filings are examined in the EC, the cells and their biofilms are intact (Figure 11). Cells remain plump/hydrated and the EPS retain moisture and appear as a continuous layer. Similar to that observed for *P. putida*, many of the *Oceanospirillum* are encrusted with electron dense material, presumably corrosion products (Figure 11). Initial attempts to acquire EELS core-loss spectra from the copper corrosion products encrusting the *Oceanospirillum* were unsuccessful, presumably because the EPS layer was sufficiently thick to obscure core loss features with background inelastic scattering.

DISCUSSION

The capability to image hydrated specimens inside a transmission electron microscope is not a new development. Since the 1970's, many groups have developed cells for transmission electron microscopes and have performed EC-TEM experiments. In the field of microbiology, Clarke *et al.* (1973) observed the production of cellular projections;[17] Parsons *et al.* (1974) visualized whole tissue culture cells;[18] Hui (1976) demonstrated movement of lipids in a membrane bilayer;[19] Fukushima *et al.* (1982) measured radiation damage to specimens;[20] Suda *et al.* (1992) examined the contraction of muscle fibers;[21] and Sugi *et al.* (1997) demonstrated the movement of the head domain of myosin molecules caused by hydrolysis of adenosine triphosphate.[22] While this in no way represents all the papers published during this period, it gives an overview of the types of experiments being carried out with an EC in the field of microbiology.

The ability to analyze the energy loss of the electron beam as it interacts with a specimen in the EC, allows elemental identification, as well as the potential for oxidation state determination and elemental mapping using energy-filtered images. Elemental maps are produced by using energy selecting slits that allow only those electrons that have loss energy corresponding to a core-loss edge of a particular element to form the image. The ability to acquire images, selected area electron diffraction patterns, EELS spectra, and potentially energy-filtered elemental maps of hydrated specimens in the EC will facilitate study of the location of metal species in bacteria and associated biopolymers.

CONCLUSIONS

In this study, the EC-TEM was used to directly observe hydrated bacteria in association with corroding iron and copper. Chemical information was acquired with high spatial resolution using EELS. In principle, it should be possible to determine the valence state of corrosion products in the EC, greatly facilitating the study of MIC.

ACKNOWLEDGEMENTS

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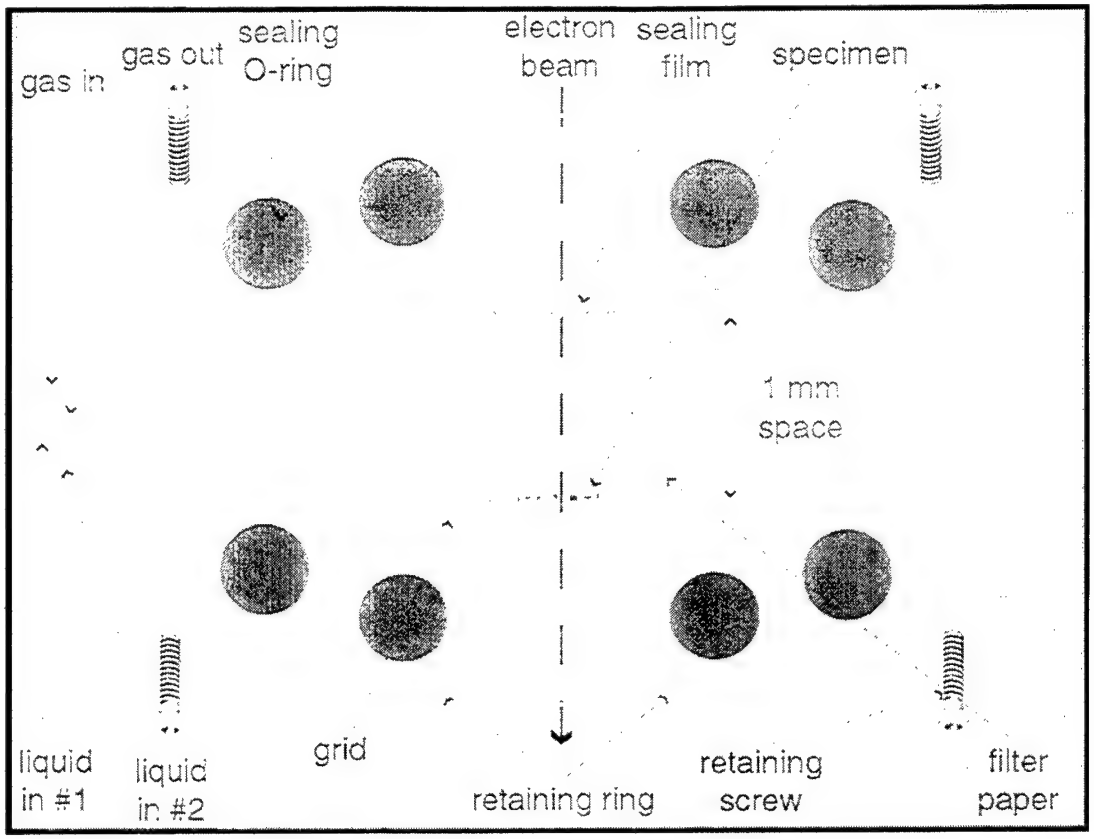

Figure 1. A diagram of EC specimen chamber for the 4-line EC *in situ* holder. Contrary to the diagram, the four lines lie in the same horizontal plane and are arranged radially around the specimen chamber, rather than the simplistic vertical arrangement shown here.

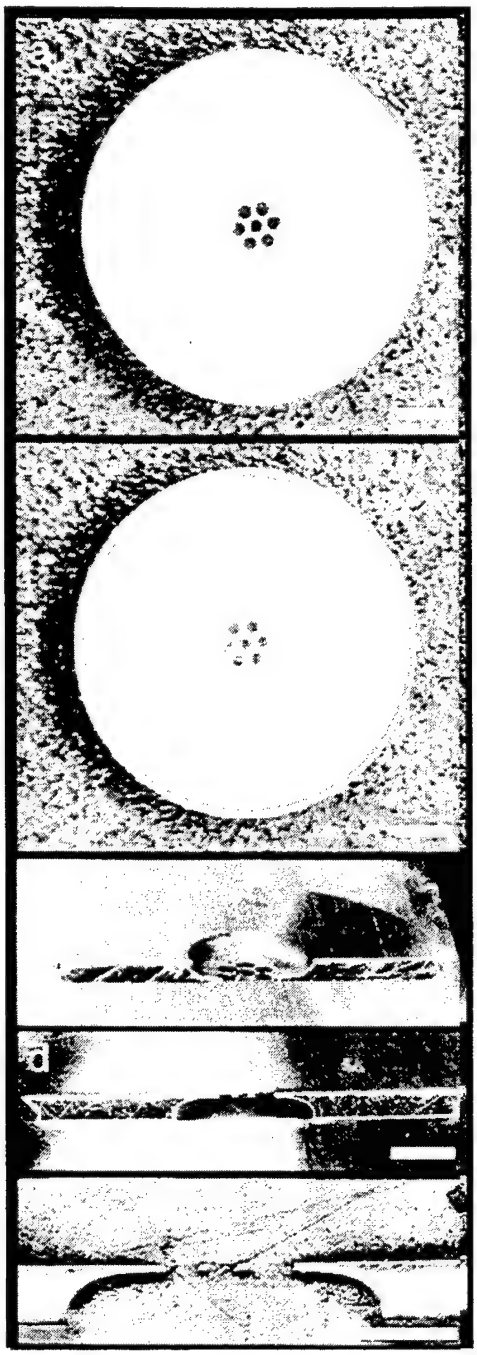

Figure 2. Seven hole EC-TEM Cu grids. (a) upper surface of grid where the a-C film window is affixed; (b) underside of grid showing concave recess; (c) cross section showing underside at an angle; (d-e) cross sections of grids showing depth of the concave recess, the size of grid holes, and the thickness of support bars. Scale bars equal 0.5 mm.

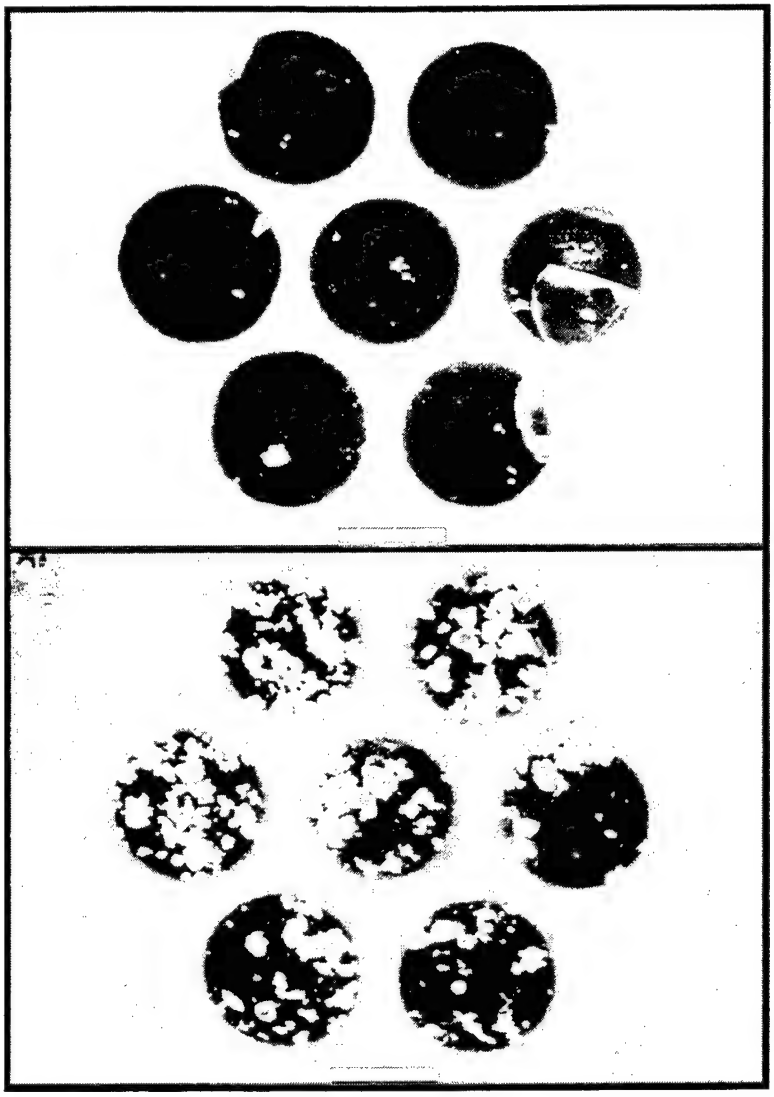

Figure 3. Images of EC-TEM windowed grids following use in the EC. (a) upper grid containing material dislodged from lower grid during course of an experiment; (b) lower grid containing corroded iron filings and bacteria. Note that the carbon support film is intact on both the upper and lower grids. Scale bar is 100 μm.

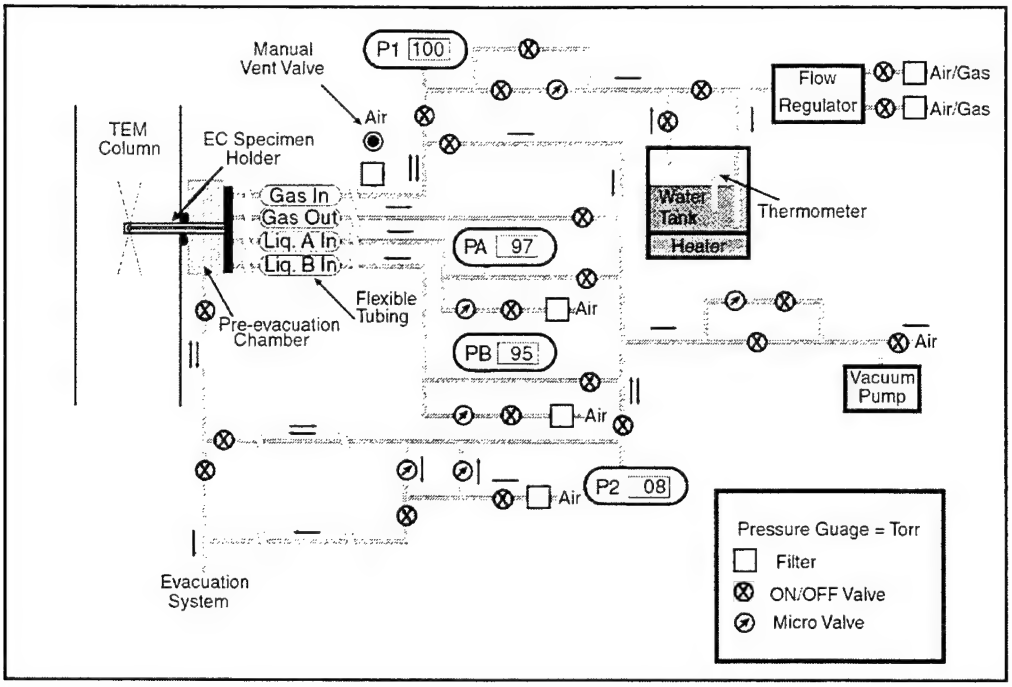

Figure 4. Diagrammatic representation of the EC control system.

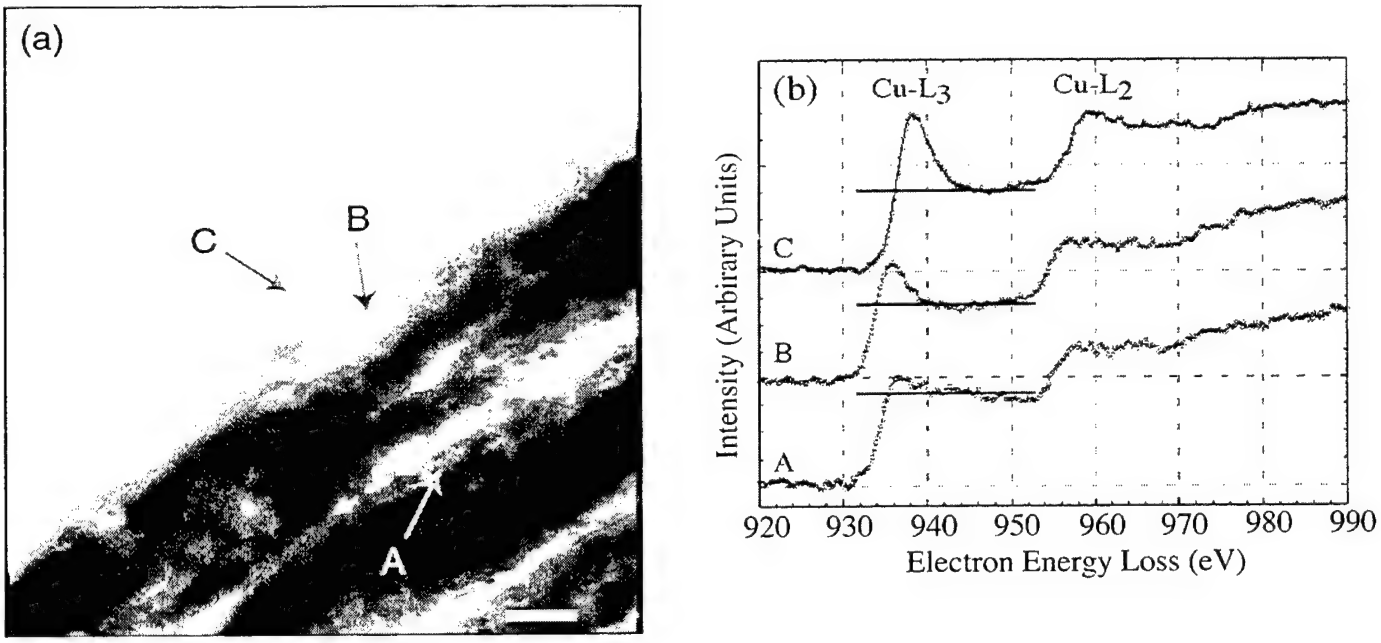

Figure 5. Copper corrosion in natural seawater. (a) conventional TEM micrograph of copper foil with associated corrosion products, scale bar is 200 nm. (b) EELS spectra from the three regions indicated in (a). The pre-edge background was subtracted from the spectra and deconvolution was used to remove plural scattering. The spectra are normalized to the post-edge background and offset from one another. The three spectra show Cu^0 at the base metal *A*, and progressively higher oxidation from *B* to *C*.

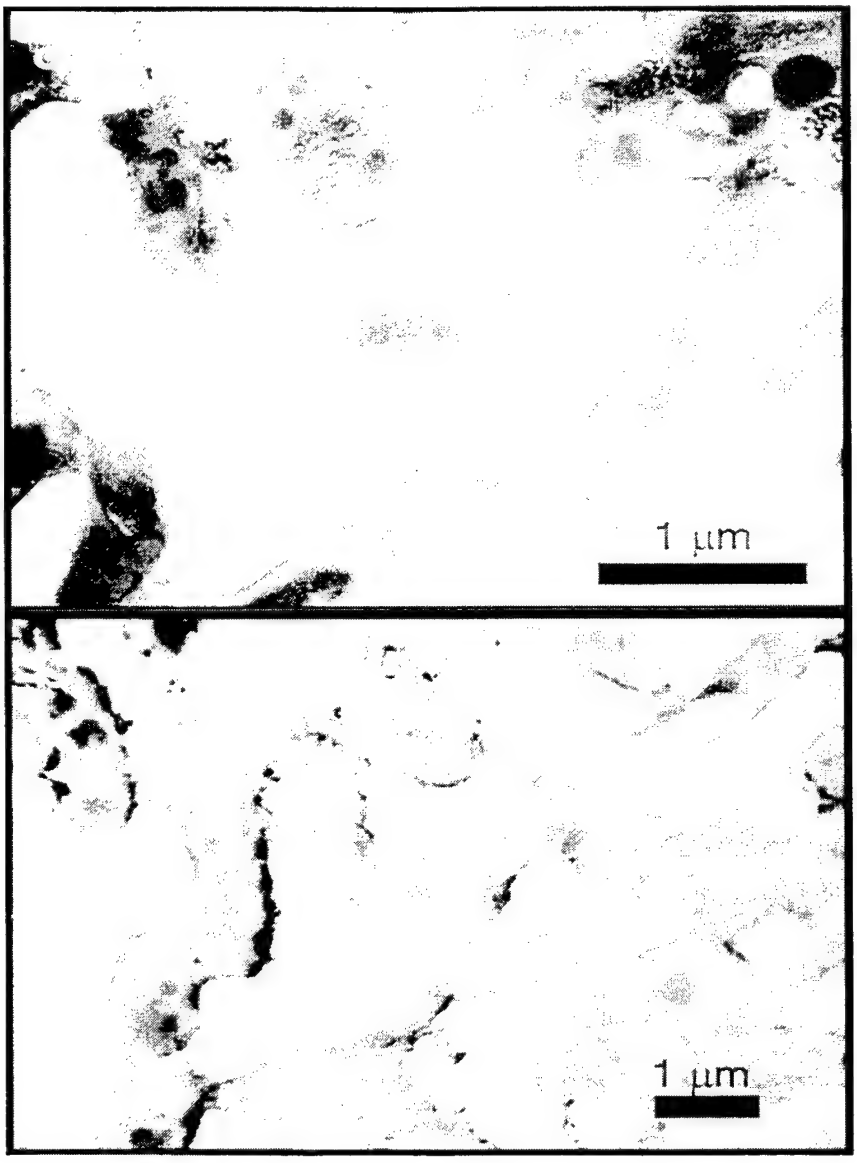

Figure 6. *Pseudomonas putida* on an a-C support film as imaged under high vacuum using conventional TEM. (a-b) dehydration and rupture of the cell membrane is apparent and the associated extracellular polymers exhibit filamentous structures between cells. Scale bar is 1.0 μm.

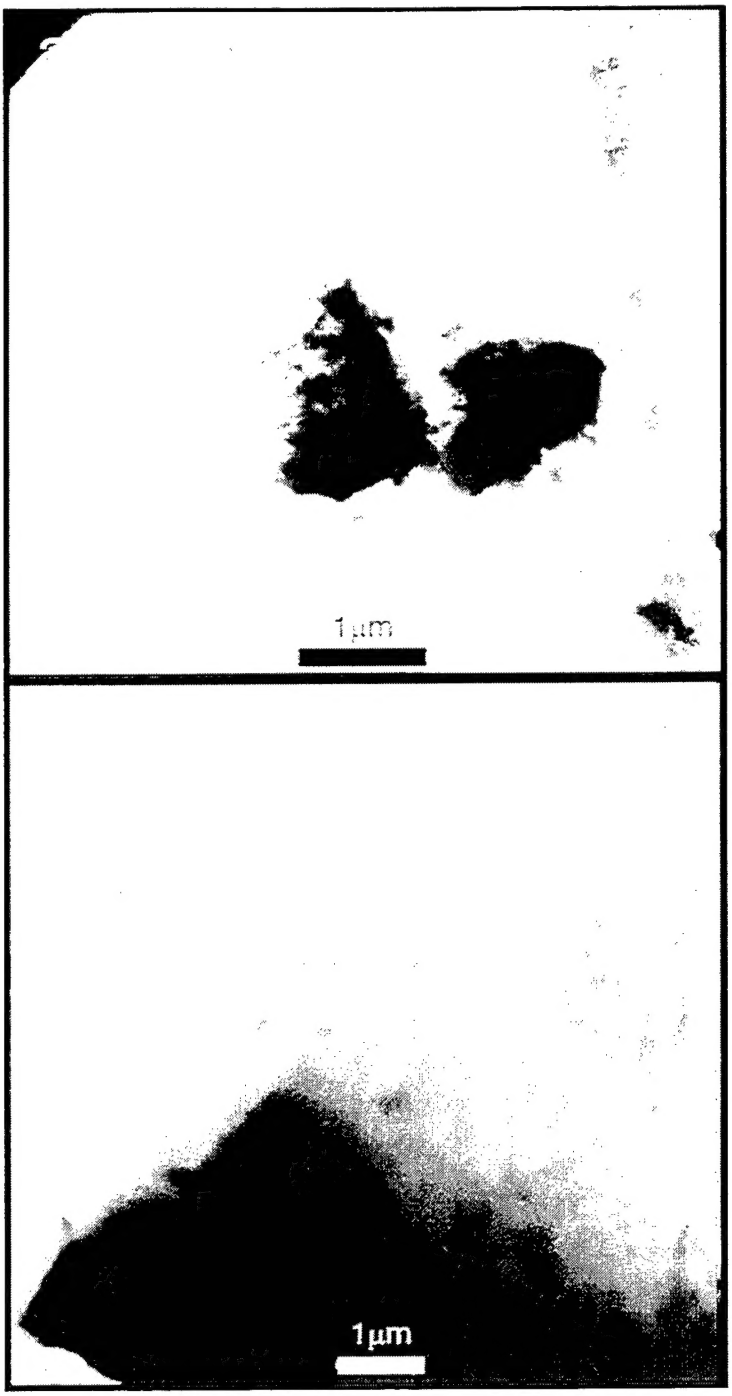

Figure 7. *Pseudomonas putida* on corroding Fe filings as imaged in the EC at 100 torr. (a-b) note the complete hydration and smoothness of the bacteria and the fluffy appearance of the extracellular polymer attaching the bacteria to the corroding Fe particle. Scale bar is 1.0 µm.

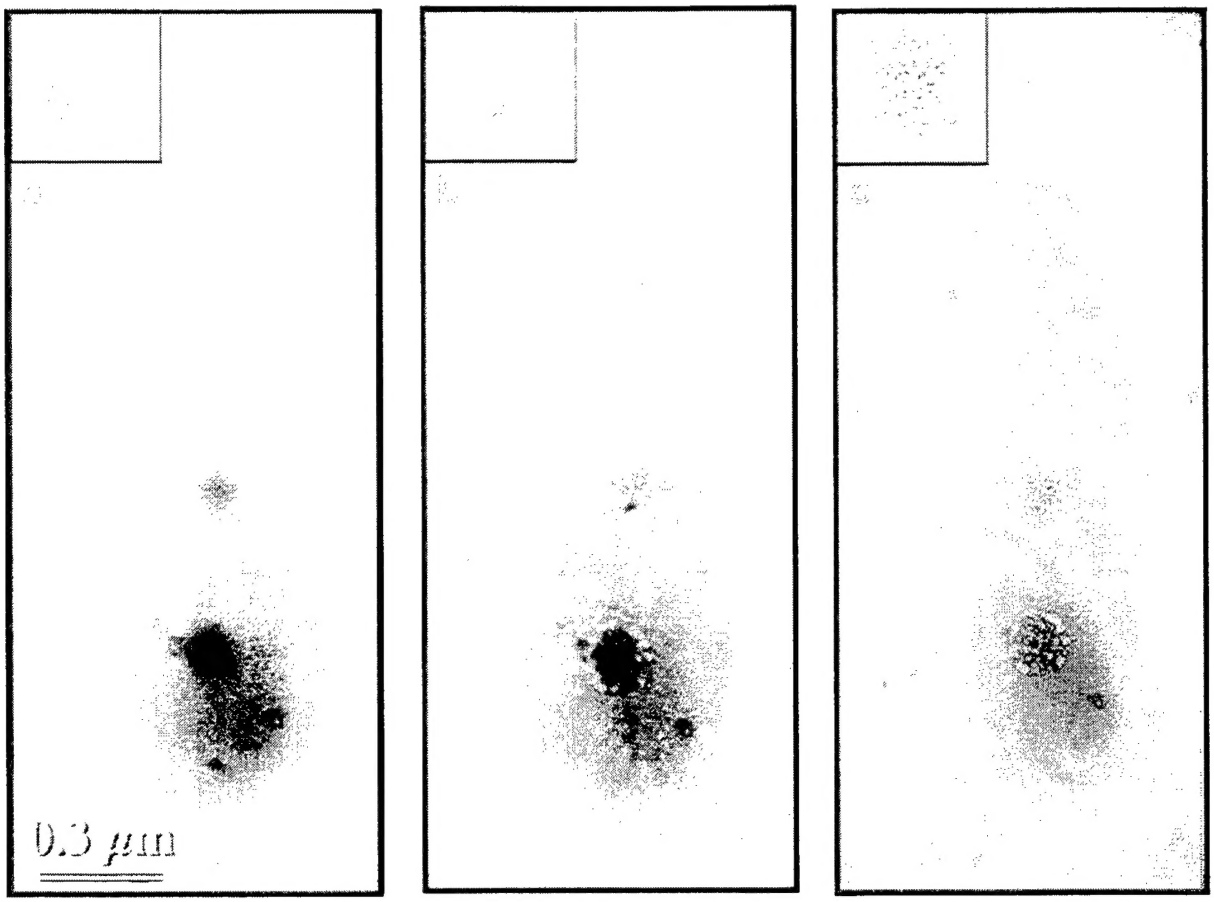

Figure 8. Series of images of hydrated *Pseudomonas putida* in the EC at 100 torr as a function of time (several minutes). Progressive electron beam damage to the nucleoid region is evident. Insert is a higher magnification of the nucleoid area.

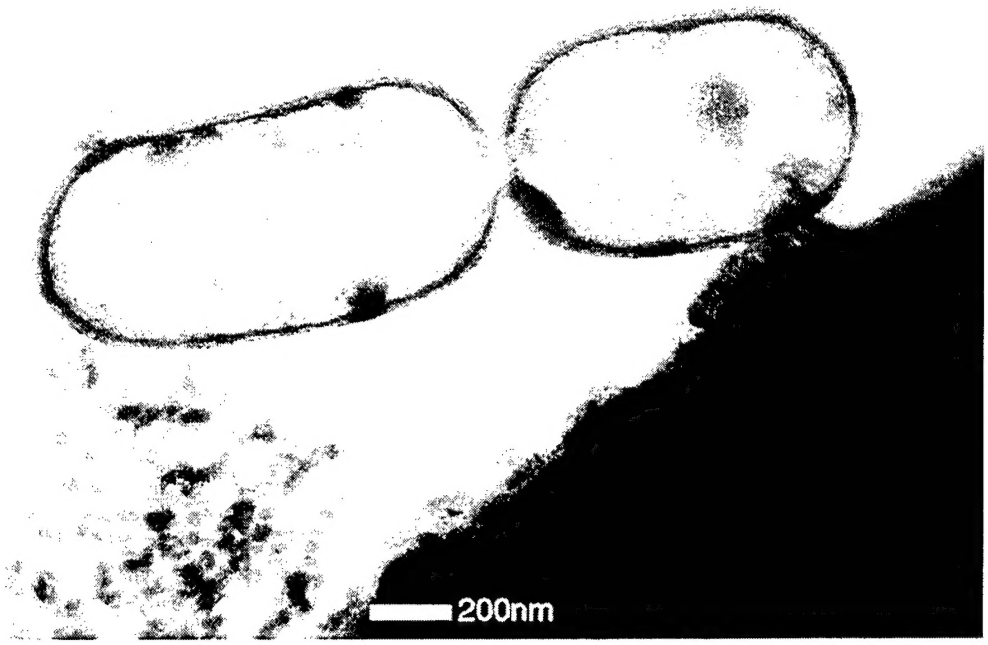

Figure 9. *Pseudomonas putida* imaged in the EC at 100 torr after removal of excess moisture by circulation of dry air through the environmental chamber. Scale bar is 200 nm.

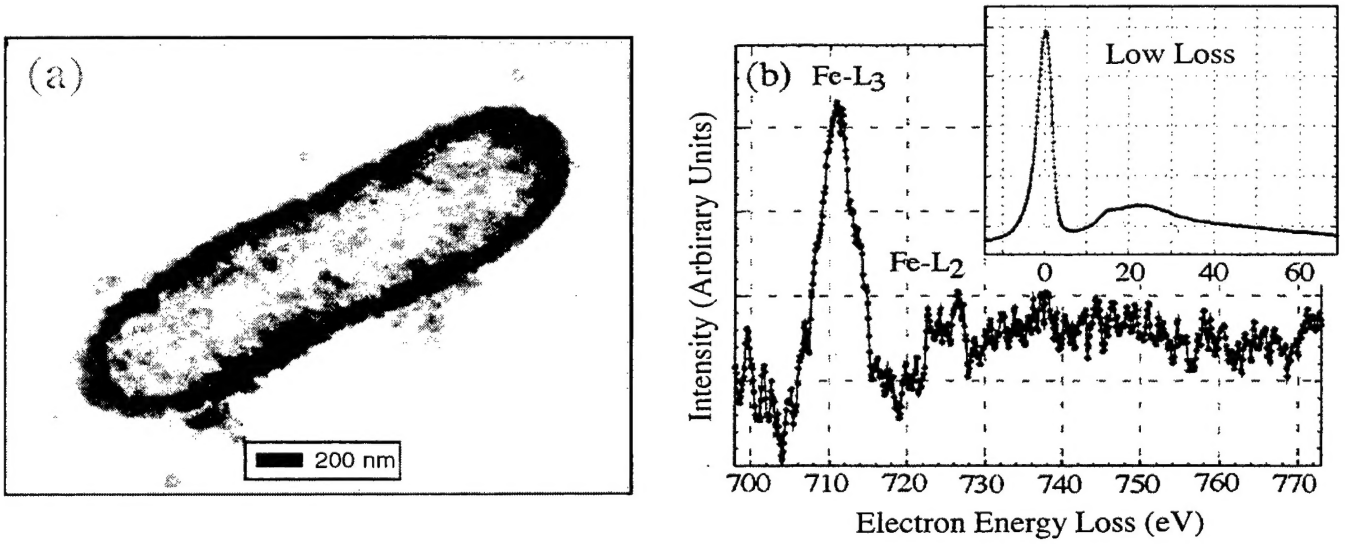

Figure 10. (a) *Pseudomonas putida* in the EC at 100 torr after removal of excess moisture by circulation of dry air through the environmental chamber. The cell is encrusted with electron dense particulates. (b) EELS spectrum from the bacteria demonstrate the presence of Fe based on the presence of the L_3 and L_2 core loss features of Fe. The core-loss regime spectrum is pre-edge background subtracted and deconvolution was used to remove plural scattering.

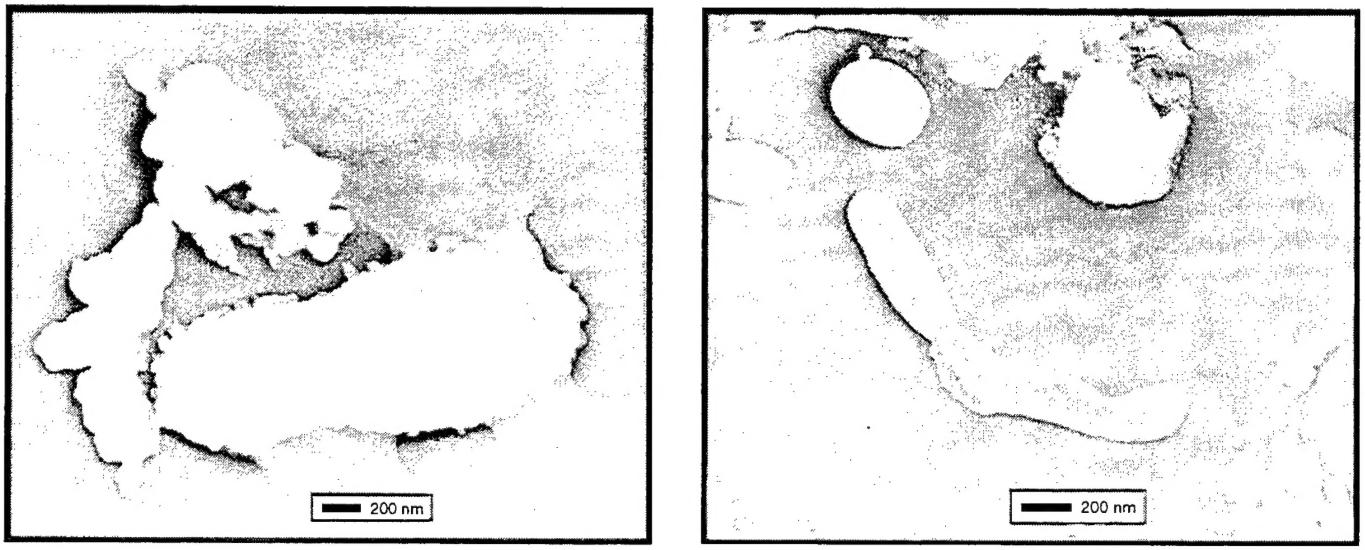

Figure 11. *Oceanospirillum* sp. on corroding copper filings in the EC at 100 torr (a) while circulating air saturated with water vapor, (b) while circulating room air. Note the complete hydration and smoothness of the extracellular polymer. Scale bar is 200 nm.